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Effects of the *tat* and *nef* Gene Products of Human Immunodeficiency Virus Type 1 (HIV-1) on Transcription Controlled by the HIV-1 Long Terminal Repeat and on Cell Growth in Macrophages

KATHLEEN M. MURPHY, MATTHEW J. SWEET, IAN L. ROSS, AND DAVID A. HUME*

Centre for Molecular Biology and Biotechnology, The University of Queensland, Queensland 4072, Australia

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The RAW264 murine macrophage cell line was used as a model to examine the role of the *tat* and *nef* gene products in the transcription regulation of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) in macrophages. Contrary to claims that the activity of the HIV-1 LTR responds poorly in rodent cells to *trans* activation by the viral *tat* gene product, cotransfection of RAW264 cells with a *tat* expression plasmid in transient transfection assays caused a >20-fold increase in reporter gene expression that was inhibited by mutations in the TAR region. RAW264 cells stably transfected with the *tat* plasmid displayed similarly elevated HIV-1 LTR-driven reporter gene activity. By contrast to previous reports indicating a negative role for *nef* in HIV transcription, cotransfection of RAW264 cells with a *nef* expression plasmid *trans* activated the HIV-1 LTR driving either a chloramphenicol acetyltransferase or a luciferase reporter gene. The action of *nef* was specific to the LTR, as expression of *nef* had no effect on the activity of the simian virus 40, c-*fms*, urokinase plasminogen activator, or type 5 acid phosphatase promoter. *trans*-activating activity was also manifested by a frameshift mutant expressing only the first 35 amino acids of the protein. The effects of *nef* were multiplicative with those of *tat* gene product and occurred even in the presence of bacterial lipopolysaccharide, which itself activated LTR-directed transcription. Examination of the effects of selected mutations in the LTR revealed that neither the κB sites in the direct repeat enhancer nor the TAR region was required as a *cis*-acting element in *nef* action. The action of *nef* was not species restricted; it was able to *trans* activate in the human monocyte-like cell line Mono Mac 6. The presence of a *nef* expression cassette in a neomycin phosphotransferase gene expression plasmid greatly reduced the number of G418-resistant colonies generated in stable transfection of RAW264 cells, and many of the colonies that were formed exhibited very slow growth. The frameshift mutant was also active in reducing colony generation. Given the absence of any effect of the frameshift mutation on *nef* function, its actions on macrophage growth and HIV transcription are discussed in terms of the role of the N-terminal 30 amino acids and of stable secondary structures in the mRNA.

The production of human immunodeficiency virus type 1 (HIV-1) viral RNA is controlled by *cis*-acting elements in the long terminal repeat (LTR), which includes a TATA box, three Sp1 sites, and an enhancer region composed of two directly repeated binding sites for the NF-κB/c-*rel* family of cellular transcription factors (1, 12, 20). Transcription of the integrated HIV-1 provirus is also regulated by viral proteins which act directly or indirectly on the viral promoter, the LTR. The best characterized of these viral regulatory proteins is the early gene product, *tat* (32). The *cis*-acting element responsive to *tat* is the TAR region located immediately downstream of the LTR transcription start site. Unlike typical enhancers, this element functions in a position- and orientation-dependent manner. The secondary structure of TAR RNA may function as a tether bringing *tat* close to the transcriptional apparatus, facilitating interaction between the *trans* activator and the promoter (2). Alternatively, there is evidence suggesting a role for *tat* in transcription elongation and nuclear export, processing, translation, or stability of HIV transcripts (27, 32).

Most of the studies of HIV LTR function in macrophages have taken place in the absence of the *tat* gene product, and the cell lines commonly used in human studies, such as the U937 promonocyte line, are relatively immature (7). Because of the availability of a great diversity of cell lines and primary macrophage sources in the mouse, and the potential power of transgenic animals for the study of the regulation of the HIV LTR, we decided to undertake an examination of the regulation of the HIV LTR in a well-characterized mouse macrophage cell line, RAW264. Transgenic animals with an integrated reporter gene linking the HIV LTR to the bacterial chloramphenicol acetyltransferase (CAT) gene expressed the reporter in T cells and macrophages (22). More recently, Corboy et al. (5) showed that the LTR of HIV-1 strains isolated from patient brain directed neuron-specific expression of *lacZ* in transgenic mice. These data suggest that HIV-1 tissue specificity is preserved across species. However, there have been several suggestions that the *tat* gene product does not *trans* activate in rodent cells (8, 32).

Of the viral regulatory gene products, *nef* is by far the most abundant early viral transcript (10, 13, 16, 26). The protein product of *nef* is a 27-kDa protein of approximately 200 amino acids transcribed from an open reading frame which overlaps the 3′ end of the *env* gene and extends into the 3′ LTR (32). It is a nonnuclear protein which undergoes posttranslational modification by myristoylation and is associated with cell membranes. Studies of the function of *nef* in HIV-1 infection in vitro have generated conflicting conclusions. There is some evidence that *nef* is a negative regulator of viral transcription (31), but others claimed that functional mutants in *nef* had no effect on viral replication (14). Still other investigations have suggested a positive function of *nef* in virus replication (18), a conclusion supported by studies of *nef* functional mutants in a simian model of AIDS (17).

* Corresponding author.

The long period of apparent viral latency in HIV-1 infection that occurs before development of disease may be due in part to low-level productive infection of mononuclear phagocytes which are resistant to the cytopathic effect of the virus and act as a virus reservoir (22). The aim of the work reported in this paper is to establish RAW264 as a useful model in which to study the regulation of the HIV LTR in macrophages. The study involves an examination of the interactions between the LTR, the *tat* and *nef* viral regulatory genes, *cis*-acting elements of the LTR, and bacterial lipopolysaccharide (LPS), a physiological regulator of macrophage function and HIV LTR-directed transcription. The results contradict claims that Tat is inactive in the mouse and suggest that *nef* could have a macrophage-specific role in the HIV-1 life cycle.

MATERIALS AND METHODS

Cells and transfection. The RAW264 murine macrophage line, obtained from the American Type Culture Collection, and the human monocyte line Mono Mac 6 (34), obtained from H. Ziegler-Heitbrock (Institute for Immunology, Munich, Germany), were maintained in RPMI 1640 medium supplemented with 5 or 10% (vol/vol) fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin in bacteriological plastic petri dishes. The fibroblast-like simian line COS-1 was obtained from the American Type Culture Collection and maintained on tissue culture plastic in Dulbecco modified Eagle medium with 10% (vol/vol) fetal calf serum. Cells were transfected by electroporation, using a Bio-Rad Gene Pulser and Capacitance Extender (set at 750 V cm^{-1} and 960-μF capacitance) at room temperature in 200 μl of medium plus 10% (vol/vol) fetal calf serum (3). CAT assays were performed as described by Gorman et al. (11) and quantitated by using an AMBIS Radioanalyser. Luciferase assays were carried out by using the GeneLight system supplied by Promega and a Bertholdt luminometer. In each case, activities were normalized to protein concentration determined by using a modified Bradford assay (Bio-Rad). Stable transfectants were generated by using the same electroporation procedure with linearized plasmids carrying the neomycin phosphotransferase (*neo*) gene. Following overnight growth in nonselective medium, 5×10^5 cells per well were distributed in a 24-well plate in 1 ml of medium with G418 (Geneticin; GIBCO-BRL) at 200 μg/ml. The selective medium was replaced every 2 or 3 days; after 10 to 14 days, clones were counted, selected, and expanded through culture in gradually increasing volumes of selective medium. All replicates involved fully independent transfections and assays of cells.

Plasmids. The Tat expression plasmid pJKC63.4.1 is a Moloney retroviral vector carrying a synthetic *tat* gene under the control of the viral LTR and a *neo* gene under the control of the simian virus 40 (SV40) early promoter (9) (a gift from Jonathan Karn, MRC Laboratory of Molecular Biology, Cambridge, England). pHIV-CAT (or pU3R-III), which carries the CAT gene under the control of the HIV-1 LTR (29), and pHIV-CATΔκB, identical to pHIV-CAT except for GGGG-to-GCTC mutations in both copies of the direct repeat (24), were gifts of Gary Nabel (Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, Mich.). pSV2CAT (11) contains the SV40 early promoter linked to CAT. The alternative HIV-1 LTR-CAT plasmid pD5.3.3 was a gift from Jonathan Karn, as were its SAS, SSA, and SAA variants containing antisense mutations of the DNA leading to changes in the secondary structure of TAR RNA in the bulge region, the loop region, and both bulge and loop regions, respectively (9). HIV-luciferase reporter plasmids were constructed by ligating an *Xho*I-to-*Hin*dIII fragment excised from pHIV-CAT or pHIV-CATΔκB into the luciferase expression plasmid pGL2-Basic (Promega) to generate pGL-LTR and pGL-LTRΔκB, respectively. A 4-bp deletion in the TAR region of pGL-LTR was created by cutting the plasmid at the unique *Sac*I site in the TAR loop region (9), removing the single-stranded ends with T4 DNA polymerase, and religating the plasmid to generate pGL-LTRΔTAR. The c-*fms* promoter plasmid pGL3.5*fms* has been described by Xie et al. (33). A urokinase plasminogen activator promoter construct was produced by ligating the 7-kb *Xba*I fragment from puPACAT2 (3) into the multiple cloning site of pGL2-Basic to produce pGL-uPA. pGL-MAP1 contains the promoter of murine type 5 acid phosphatase gene described in reference 4. In pGL2-Control (Promega), the luciferase gene is under the control of the SV40 early promoter and enhancer. Nef expression plasmids were pNEF, containing the *nef* coding region extending only four nucleotides 3′ of the *nef* stop codon under the control of the cytomegalovirus (CMV) immediate-early promoter, pNEF-fs, a version of this plasmid with a frameshift at the *Xho*I site, and pNEF-LTR, the same vector with additional 3′ untranslated U3 and R sequence up to the *Hin*dIII site (14) (gifts of Stephen Hammes, Howard Hughes Medical Institute, Duke University Medical Center, Durham, N.C.). The *neo* expression plasmid pMC1NeoPolyA was obtained from Stratagene. The *nef* expression plasmids pNEF-Neo and pNEF-fs-Neo were constructed by blunt-ended ligation of the *Xho*I-*Hin*dIII *neo* fragment of pMC1NeoPolyA into the *Eco*RI site of pNEF and pNEF-fs. The control CMV promoter expression plasmid was pcDNA1 (Invitrogen).

Reagents. Bacterial LPS from *Salmonella minnesota* R595, obtained from List Biological Laboratories (Campbell, Calif.), was dissolved by sonication in 0.1% triethylamine.

RESULTS

Tat and HIV-1 LTR-directed transcription in RAW264 cells. The HIV-1 LTR reporter construct pHIV-CAT transiently transfected into RAW264 cells produced substantial CAT activity (Fig. 1A), but the activity was elevated still further, up to 20-fold, in stable transfectants of RAW264 cells expressing the HIV-1 *tat* gene product. A representative *tat*-transfected clone is compared with the parent line in Fig. 1A. *trans* activation in the *tat*-producing clones was specific for the HIV-1 LTR in that no effect was seen on the SV40 early promoter in the positive control vector pSV2CAT (Fig. 1A). All *tat*-transfected clones that were positive for *trans* activation appeared to be slower growing than RAW264, as assessed by a 30 to 50% decrease in [^{3}H]thymidine incorporation (data not shown), but there were no obvious effects on cell morphology.

Previous reports have suggested that *trans* activation by *tat* is restricted to human and primate cells. *trans* activation of the LTR by *tat* requires sequences in the TAR region and is believed to involve an interaction between the activator and a stable stem-loop structure formed in nascent TAR RNA (9). Hence, we decided to confirm that the response to *tat* in the RAW264 cells was mediated via the *tat* activation region of TAR. In Fig. 1B, mutations predicted to alter the secondary structure of TAR RNA resulted in changes to basal expression from the LTR in RAW264 cells. Mutation of the bulge or loop alone (SAS, SSA [9]) halved basal expression. The double mutation, both bulge and loop (SAA), reduced basal expression by eightfold. The effect on *tat*-stimulated expression was more marked; 9- and 5-fold reductions were induced by the SAS and SSA mutants, respectively, and the double mutation causing a 30-fold reduction (Fig. 1B). The ability of *tat* to *trans*

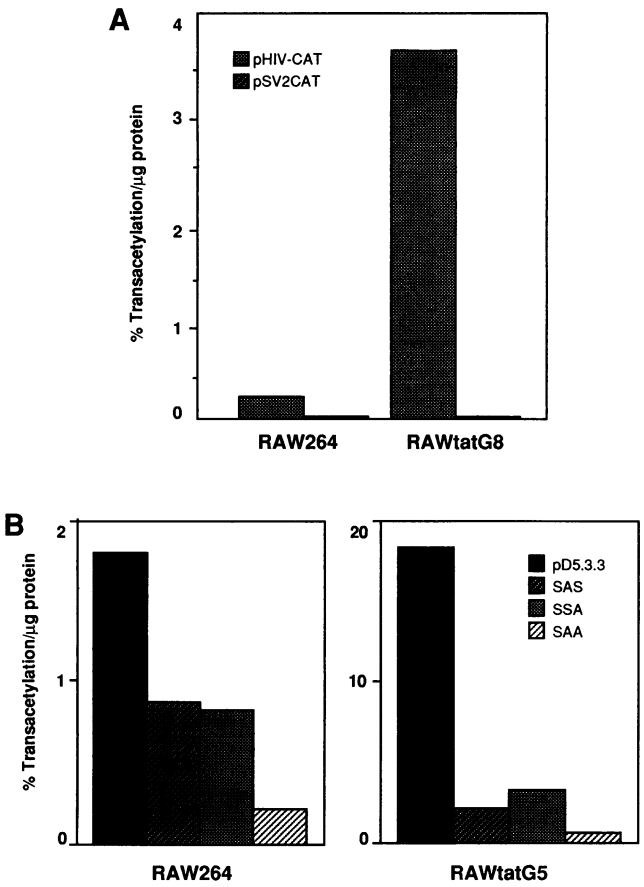

FIG. 1. (A) The activity of the HIV-1 LTR in control and Tat-producing RAW264 murine macrophage cell lines. RAW264 cells were transfected with the expression vector pJKC63.4.1, which has a *neo* gene as well as a *tat* expression cassette (9). Control RAW264 cells and a representative *tat*-expressing cell line, RAWtatG8, were transfected with 10 μg of pHIV-CAT or pSV2-CAT by electroporation as described in Materials and Methods. After 48 h, the cells were harvested and assayed for CAT enzymatic activity. Values are averages of two independent transfections. Results for RAWtatG8 are representative of results for four other *tat*-expressing clones analyzed (see also RAWtatG5 in panel B). (B) Effects of mutants in the TAR region of the HIV-1 LTR on basal and Tat *trans*-activation activity in RAW264 cells. Control RAW264 or *tat*-expressing RAWtatG5 cells were transfected by electroporation with wild-type HIV-1 LTR reporter plasmid pD5.3.3 and derivatives of pD5.3.3 with antisense mutations in the TAR sequence predicted to cause alterations in the structure of the RNA stem-loop in nascent mRNA. Plasmid SAS is altered in the bulge region in the TAR upper stem, SSA is altered in the loop region, and SAA is mutated in the both the bulge and loop regions (9). After 24 h, the cells were harvested and CAT enzyme activity was assayed. The results are averages of duplicate independent transfections and are typical of results of three experiments.

activate in the murine macrophages was also observed when the expression plasmid was cotransfected with either the HIV-CAT reporter construct (Table 1) or an HIV-luciferase reporter plasmid (Table 2) in transient transfections. In the latter case, a mutant LTR in which a 4-bp deletion of the TAR region had been made (pGL2-LTRΔTAR) was almost completely unresponsive to *tat*. Taken together, the data indicate that the effect of *tat* in the stably transfected cell lines and in transient transfections is specific to LTR-driven reporter constructs with an intact TAR region and that there is no significant difference between the RAW264 murine macrophage cell line and human and simian lines reported in the literature in terms of responsiveness to *tat*.

TABLE 1. Comparison of the effects of cotransfection with Nef and Tat expression plasmids on the activity of the HIV-1 LTR in RAW264 murine macrophages and COS-1 simian fibroblasts[a]

Cotransfected plasmid(s)	CAT activity (% transacetylation/μg of protein/h)[b]	
	RAW264	COS-1
None	0.18	0.52
Control vector	0.14	ND
Tat	5.80	99.1
Nef	0.70	0.27
Tat + Nef	15.8	75.7
Tat + control vector	ND	83.4

[a] Cells were transfected by electroporation with 2.5 μg of pHIV-CAT reporter together with 2.5 μg of pNEF (Nef expression plasmid), pJKC63.4.1 (Tat expression plasmid), and/or a control plasmid (pcDNA1) as indicated, and CAT activity was assayed 48 h later.

[b] Mean of duplicate transfections varying by less than 10% from the mean. The transfections of RAW264 and COS-1 cells were performed in parallel, using the same reagents. The experiment is representative of three. ND, not determined.

Effects of Nef on transcription directed by the HIV LTR. As noted in the introduction, *nef* has variously been attributed negative or negligible effects on transcription directed by the HIV LTR. When pHIV-CAT was transfected into RAW264 cells together with a *nef* expression plasmid, either pNEF or pNEF-LTR, which contains more of the 3′ LTR, there was a three- to fivefold increase in CAT activity (Fig. 2A; Table 1). The increase in CAT expression induced by the *nef* plasmids was not dependent on the production of a full-length protein, since pNEF-fs, which encodes a 46-amino-acid protein of which the initial 35 correspond to the N terminus of *nef*, was

TABLE 2. Effects of mutations in the direct repeat (ΔκB) and TAR (ΔTAR) regions of the HIV-LTR on basal activity and responsiveness to bacterial LPS, Nef, and Tat[a]

Reporter	Cotransfected plasmid(s)	Luciferase activity (RLU/μg of protein)[b]	
		Control	+LPS
pGL-LTR	Control	9,400	20,700
	Tat	81,400	99,300
	Nef	23,400	33,600
	Tat + Nef	194,300	257,500
pGL-LTRΔκB	Control	1,600	3,400
	Tat	11,600	24,500
	Nef	3,100	8,800
	Tat + Nef	30,000	64,300
pGL-LTRΔTAR	Control	11,900	23,000
	Tat	19,400	32,400
	Nef	35,800	55,400
	Tat + Nef	54,600	112,000

[a] RAW264 cells were cotransfected with 10 μg of HIV LTR-luciferase reporter plasmid (pGL-LTR; wild type or mutant as indicated) plus PJKC63.4.1 (the Tat expression plasmid; 2 μg) or pNEF (Nef expression plasmid; 10 μg). Control vector plasmid (pcDNA1) was added as required to equalize quantities of transfected DNA to a total of 22 μg per transfection. After 24 h, cells were harvested and assayed for luciferase activity.

[b] Average of independent duplicate transfections varying by less than 10% around the mean. The experiment is representative of three. RLU, relative light units.

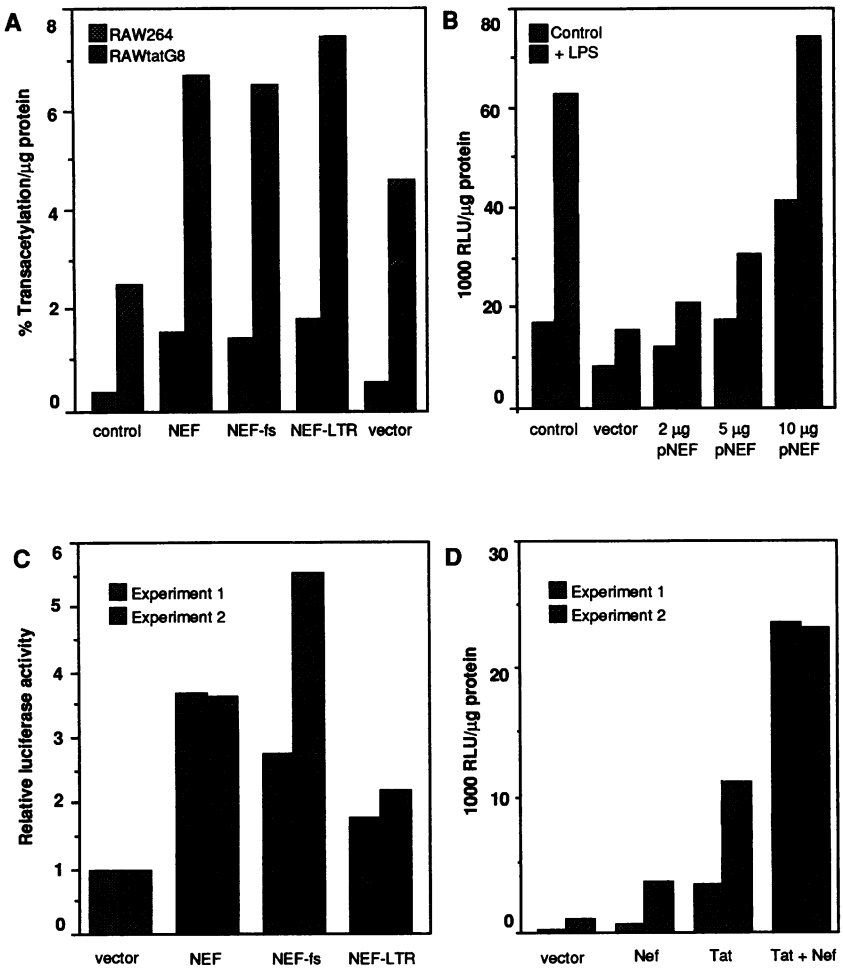

FIG. 2. (A) *trans* activation of the HIV-1 LTR in RAW264 cells by the *nef* gene. RAW264 cells or *tat*-expressing RAWtatG8 cells were cotransfected with 10 μg of pHIV-CAT and either pNEF, pNEF-fs, pNEF-LTR, or a control CMV promoter vector (pcDNA1) at a 2:1 molar ratio by electroporation as described in Materials and Methods. After 48 h, CAT activity was assayed and is expressed as percent transacetylation per microgram of protein extract assayed. Values shown are means of either triplicate transfections or two sets of duplicates. The experiment is representative of three except that the small stimulus observed in response to pcDNA1 was not observed elsewhere. (B) Effect of LPS on the dose dependence of *trans* activation of the HIV-1 LTR in RAW264 cells by *nef*. RAW264 cells were cotransfected with 10 μg of pGL-LTR and 2, 5, and 10 μg of pNEF. Total DNA transfected was made up to 20 μg with the CMV promoter expression vector pcDNA1. Each transfection mixture was divided into two, and half of the cells were treated with 500 ng of LPS per ml. After 24 h, the cells were harvested and assayed for luciferase activity. Results are averages of two independent series of duplicate transfections. (C) *trans*-activating effects of *nef* in Mono Mac 6 cells. Mono Mac 6 human macrophages were cotransfected with 10 μg of pGL-LTR and 10 μg of either pNEF, pNEF-fs, pNEF-LTR, or a control CMV promoter vector (pcDNA1) by electroporation as described in Materials and Methods. After 24 h, cells were harvested and assayed for luciferase activity. Two sets of results from independent experiments are shown, both normalized for reporter-only luciferase activity. Values are averages of duplicate transfections varying by less than 10% from the mean. (D) Additive responses to *nef* and *tat* in Mono Mac 6 cells. Mono Mac 6 human macrophages were cotransfected with 10 μg of pGL-LTR and one or both of Tat expression plasmids pJKC63.4.1 (2 μg) and pNEF (10 μg). CMV vector DNA (pcDNA1) was added as required to equalize quantities of transfected DNA to 22 μg in total. After 24 h, cells were harvested and assayed for luciferase activity. Results from two independent experiments are shown. Values are averages of independent duplicate transfections varying by less than 10% from the mean. RLU, relative light units.

equally effective as a *trans* activator. The response of RAW264 macrophages to the *nef* expression plasmids could not be attributed to transfection with the CMV promoter in the expression plasmid. Transfection with a control plasmid alone sometimes led to an increase of at most 1.5-fold in CAT expression controlled by the LTR (as seen in Fig. 2A but not evident in the more typical experiment in Table 1). *trans* activation by *nef* was equally evident in *tat*-stable transfectants of RAW264 (Fig. 2A) and when it was cotransfected transiently with the *tat* expression plasmid (Table 1), indicating that the actions of the two viral gene products are multiplicative. Finally, the effects of *nef* were also evident when the expression plasmid was cotransfected with an alternative reporter, pGL-LTR, in which the HIV LTR was placed upstream of the luciferase gene. A dose-response curve demonstrating increasing luciferase activity in response to increasing amounts of *nef* expression plasmid is shown in Fig. 2B.

Because the actions of both *tat* and *nef* in RAW264 contrasted with previous reports, with respect to either tissue specificity or the nature of the response, comparative transient cotransfection studies were performed in RAW264 murine macrophages and COS-1 simian fibroblasts, in which transcrip-

TABLE 3. Specificity of *trans* activation of the HIV-LTR by Nef in RAW264 murine macrophages[a]

Promoter	Luciferase activity (RLU/μg of protein)[b]		Fold change
	Control	+Nef	
HIV LTR	1,900	10,200	5.4
SV40 promoter/enhancer	13,800	19,500	1.4
Urokinase	400	350	0.9
Type 5 acid phosphatase	650	900	1.4
c-*fms*	65	60	0.9

[a] RAW264 cells were transfected by electroporation with 10 μg of reporter construct in which the luciferase reporter gene was under the control of the promoters indicated together with 10 μg of either control vector (pcDNA1) or the Nef expression plasmid pNEF. Details of the reporter plasmids are provided in Materials and Methods. After 24 h, the cells were harvested and luciferase activity was determined as described in Materials and Methods.

[b] Average of two independent experiments. RLU, relative light units.

TABLE 4. Effect of Nef on the generation of stable transfectants of RAW264 cells[a]

Plasmid	Relative frequency of stable transfectants			
	Expt 1	Expt 2	Expt 3	Expt 4
pNEF (*Hin*dIII)	0.82			1.13
pNEF (*Kpn*I)				0.99
pNEF (*Ssp*I)			0.22	0.22
pNEF (*Bst*EII)	0.04			0.09
pNEF-fs (*Ssp*I)			0.54	
pNEF-fs (*Hin*dIII)		2.08	1.40	
pNEF-fs (*Kpn*I)		1.31	1.02	
pNEF-fs (*Bst*EII)	0.10	0.12	0.17	

[a] Cells were transfected with 2.5 μg of either a wild-type (pNEF) or frameshift mutant (pNEF-fs) Nef expression plasmid also containing the Neo-resistance cassette from pMC1NeoPolyA (see Materials and Methods). After 24 h, G418 was added at 200 μg/ml. G418-resistant colonies (>50 cells) were counted after 14 days in culture. Prior to transfection, the expression plasmids were linearized with different restriction enzymes as indicated in brackets. *Ssp*I cuts outside both the Nef and Neo expression cassettes. The *Hin*dIII site lies between the CMV promoter and the *nef* start codon. *Bst*EII cuts between the 3′ untranslated region of *nef* and the polyadenylation signal encoded by the vector and, *Kpn*I cuts 226 bp into the *nef* coding sequence. Results are expressed relative to the frequency of Neo-resistant colonies arising from transfection with the linearized control plasmid, pMC1NeoPolyA, which varied from 72 to 201 colonies per 10^6 cells. In each of four independent experiments shown, the results are averages of duplicate independent transfections.

tional repression by *nef* had been observed (14). By contrast to the fourfold *trans* activation seen in RAW264 cells transfected in parallel, *nef* caused a slight repression in COS-1 cells that was only marginally greater than that caused by the parent vector (Table 1). We have seen no fundamental species difference in the activity of the HIV LTR in macrophages, but the effect of *nef* was so unexpected that it became important to confirm the positive effect of *nef* on transcription in a human macrophage line. Using the luciferase reporter plasmid for greater sensitivity, we examined the effects of *nef* in the human monocyte cell line Mono Mac 6 (Fig. 2C). pNEF and pNEF-fs both increased LTR-driven reporter gene expression three- to fivefold in Mono Mac 6 cells. pNEF-LTR was somewhat less active, perhaps as a result of promoter competition between the extended LTR in the expression plasmid and the reporter plasmid (14). As in RAW264 murine macrophages, the effects of cotransfection with *nef* and *tat* expression plasmids in Mono Mac 6 cells were multiplicative (Fig. 2D). These data indicate that the actions of *tat* and *nef* are not species specific. Because RAW264 cells are more readily transfected than Mono Mac 6 cells, further delineation of the mechanism of action of *nef* in macrophages was carried out in the mouse system.

***cis*-acting elements involved in *trans* activation of the HIV LTR by *nef* and bacterial LPS.** Bacterial LPS has been implicated as a physiological regulator of HIV-1 transcription in monocytes/macrophages (25). Figure 2B shows that in RAW264 cells transfected with pGL-LTR, treatment with LPS caused a *trans* activation that was similar in magnitude to the effect of *nef*. The effect of LPS was additive with the action of *nef*. This finding indicates that LPS and *nef* act via different pathways and eliminates any possibility that the action of the *nef* expression plasmid is due to contamination with LPS or any compound with a similar mode of action. Table 3 provides additional evidence for the specificity of the effect of *nef* for the HIV LTR. Cotransfection with *nef* had no effect on the activity of the SV40 promoter/enhancer (which is LPS inducible [unpublished data]), the urokinase plasminogen activator promoter (which can be induced by phorbol esters and growth factors [3]), or the macrophage-specific c-*fms* (33) and type 5 acid phosphatase (4) promoters.

Two major *cis*-acting elements have been implicated in the regulated transcription of the HIV LTR: the direct repeat enhancer containing binding sites for the NF-κB transcription factor family and the TAR sequence mentioned above. LPS has been reported to induce NF-κB in macrophages and to mediate some of its effects on HIV transcription via this pathway (20, 25). A reporter construct containing mutations in the direct repeat that would abolish NF-κB binding (pGL-LTRΔκB) was sixfold less active than the parent plasmid in RAW264 cells but was *trans* activated to the same extent by *tat*, *nef*, and LPS (Table 2). Again, the effects of the three agents were multiplicative. The basal activity of the HIV LTR was unaffected by a 4-bp deletion of the TAR sequence around the loop in the predicted stem-loop structure formed in TAR RNA (pGL-LTRΔTAR), but as noted above, *tat* stimulation was almost eliminated. This mutation also had no effect on the actions of LPS and *nef*, which together caused a 10-fold *trans* activation of the mutated reporter construct. Note that the failure of the TAR deletion to alter basal activity differs from the inhibitory effect of the TAR antisense mutations shown in Fig. 1B, suggesting that the latter mutations act on basal expression independently of their potential effects on RNA secondary structure. These findings suggest that the *cis*-acting elements involved in the actions of *nef* function independently of those required for a response to LPS and *tat*.

Stable transfection of RAW264 cells with *nef* expression plasmids. Because the *nef* gene product is a nonnuclear protein, we considered it likely that its ability to increase the transcriptional activity of the LTR in RAW264 cells occurred indirectly as a result of an alteration in cell function. The cotransfection studies described above eliminated the possibility that *nef* altered expression of other genes that are macrophage specific or inducible in RAW264 cells. To analyze the actions of *nef* on other aspects of macrophage function, we attempted to produce stable transfectants of RAW264 cells expressing *nef*. RAW264 were transfected with the selectable plasmid pMC1NeoPolyA or with pNEF-Neo or pNEF-fs-Neo. In side-by-side comparisons, substantially fewer G418-resistant colonies were generated when plasmids with functional expression of either Nef and Nef-fs were used as opposed to pMC1NeoPolyA (Table 4). Many of the colonies that were formed were generally smaller; they reached a moderate size and then stopped growing. Figure 3 shows an example of such a colony, which maintained a constant size after 7 weeks in culture in selective medium. The cells within the colony appear

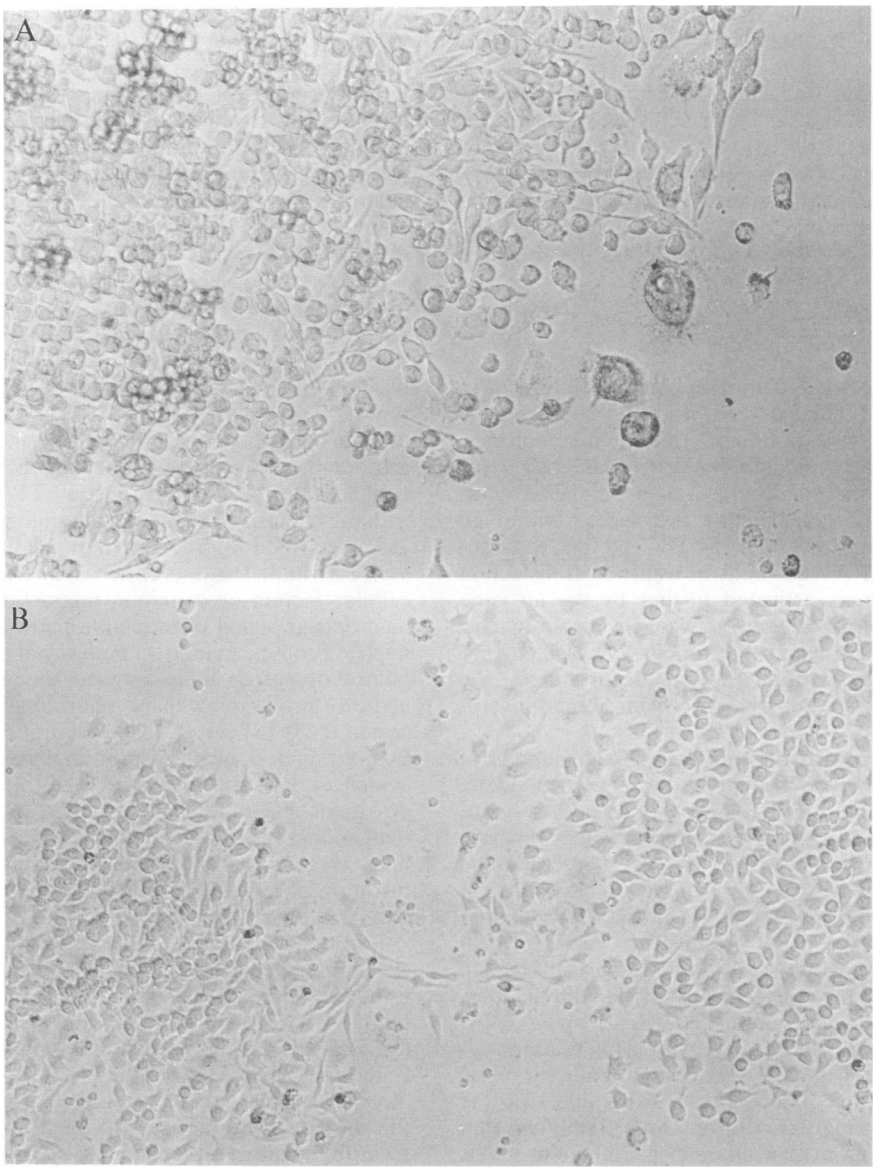

FIG. 3. The effect of *nef* on the generation of G418-resistant colonies in RAW264 cells transfected with a *neo* cassette. (A) G418-resistant cells that form one quadrant of a colony of RAW264 transfected with pNEF-Neo. The colony originated from a focus comprising about 20 cells arising from a foundation clone by 7 days after transfection. It had expanded to this size after a further 41 days in G418 medium which was replaced at 3- to 4-day intervals. (B) Untransfected RAW264 cells growing in nonselective medium.

smaller, clumped, and more rounded and with less tendency to spread on the culture dish. These data suggested that expression of Nef and Nef-fs was cytotoxic and/or caused cytostasis.

To confirm that the cytotoxic or cytostatic effects of the *nef* expression plasmids were attributable to gene products of the plasmids rather than the plasmid DNA per se or some alteration to the *neo* gene, the plasmids were linearized with restriction enzymes at different sites. The control plasmids that were ineffective at generating colonies had been linearized at the *Ssp*I site, which leaves the *neo* and *nef* cassettes intact. A single *Hin*dIII site is located between the CMV promoter and the sequences coding for Nef and Nef-fs. Hence, digestion with this enzyme separates the *nef* gene from the promoter and should prevent its transcription. Both pNEF-Neo and pNEF-fs-Neo were as active as pMC1NeopolyA at generating G418-resistant colonies when digested with *Hin*dIII, demonstrating that the effects observed are not due to the different environment of the *neo* cassette. Digestion at the *Kpn*I site, 226 bp downstream from the Nef start codon, also abolished the inhibitory actions of both the control and frameshift expression plasmids (Table 4). By contrast, digestion at the *Bst*EII site, located downstream of the Nef coding sequence but 5′ to the heterologous rat preproinsulin polyadenylation signal, did not prevent the inhibitory effects of the Nef and Nef-fs plasmids.

The inhibitory effects of *nef* did not totally preclude colony formation. A selection of G418-resistant clones that did grow were expanded, RNA was extracted, and expression of *nef* transcript was examined by Northern (RNA) analysis. None of the clones that were expanded expressed detectable *nef* mRNA (data not shown). When selected clones were exam-

ined by Southern blotting, clones derived from transfection with *Hin*dIII-cut plasmid contained both *nef* and *neo* DNA, whereas clones that might have been expected to express *nef* contained no *nef* DNA. These results suggest that the only way that a viable Neo^r line can be generated by using Nef-neo plasmids is if the *nef* expression cassette is either interrupted by digestion prior to transfection or eliminated from the DNA inserted into the genome. In summary, we were unable to generate stable Nef-expressing RAW264 cell lines, but the data indicate that Nef interferes with pathways that regulate cell growth.

DISCUSSION

This report is concerned with the complex interactions between extracellular regulators and viral and cellular gene products that regulate transcription of HIV-1 in macrophages. The murine macrophage cell line RAW264 was chosen for study because it responds to a wide range of physiological modulators and can be conveniently transfected with high efficiency (3). We have shown that the HIV LTR is constitutively highly active in RAW264 cells in transient transfection analysis and can be *trans* activated further by the *tat* gene product. The ability of *tat* to *trans* activate in mouse macrophages clearly contradicts claims that rodent cells lack cellular proteins that collaborate with *tat* (8, 28). In our studies, the activity of *tat* did not distinguish murine from human macrophages (compare Tables 1 and 2 and Fig. 2D).

The results obtained with the antisense TAR mutants of HIV-CAT were qualitatively similar to those seen in HeLa cells transfected with the same plasmids (9). In both systems, the SAA double mutant caused the greatest effect on both basal activity and *tat trans* activation (Fig. 1B). Direct binding analysis of RNA produced from this mutant suggested that it could not be recognized by *tat* (9). However, the TAR deletion mutant studied in the experiment presented in Table 2, which should completely disrupt the stem-loop structure formed in TAR RNA, had a much more selective effect than did the SAA mutant on *tat trans* activation of the HIV-LTR in RAW264 cells, causing little change in basal activity. This finding suggests that the effect of the SAA double mutant on basal promoter activity operates independently of its effect on the formation of RNA secondary structures. One possibility is that in addition to its function as a *tat* response element, TAR contains a conventional *cis*-acting enhancer element bound by a nuclear DNA-binding protein(s). RAW264 cells do contain nuclear DNA-binding proteins that interact specifically with the TAR region in electrophoretic mobility shift assays (2a), and the effects of the mutations and deletions studied herein on the binding activity of these proteins are currently being studied. Alternatively, the mutations, which would be contained in the 3′ untranslated region of the mRNA produced, might affect RNA processing or translation.

The roles of the *nef* gene are controversial, and considerable uncertainty exists regarding its function in the cycle of infection by HIV-1. Expression of a functional *nef* comprising all regions of the native protein appears essential to the disease state in the closely related simian immunodeficiency virus (SIV). An SIV clone with a deletion in the *nef* gene was infectious in macaques but caused no disease and actually protected infected individuals against challenge with pathogenic wild-type SIV (6). Conversely, Nef protein is recognized as a major inducer of cytotoxic T-cell activity in HIV (19). Kestler et al. (17) suggested that SIV *nef* was required for maintaining high virus loads during the course of persistent infection in vivo but had no effect on replication of the virus in vitro. One question that arises is whether the assays carried out in vitro have involved appropriate cells. There is strong evidence that cells of the monocyte/macrophage lineage are major reservoirs of infectious HIV. Infection of these cells may lead either directly or indirectly to many of the symptoms of AIDS, including dysfunctions of the central nervous system and gastrointestinal tract (22, 32). One possible explanation for the effects of *nef* deletions on the course of SIV infection is that *nef* plays a unique role in macrophages. We have presented data to support that view.

First, we showed that cotransfection of a *nef* expression plasmid with pHIV-CAT or pGL-LTR into mouse or human macrophage cell lines led to induction of reporter gene expression. This was not seen in the widely studied COS-1 simian fibroblast line. Our finding stands in complete contrast to other reports on the action of *nef* (14, 31), but these studies did not involve macrophage cell lines. The increase in LTR activity attributable to *nef* was independent of the direct repeat enhancer or TAR regions of the LTR and was multiplicative with the effects of Tat and the physiological regulator LPS (Fig. 2; Tables 1 and 2). The apparent cell lineage restriction of *nef trans* activation suggests either an interaction with macrophage cellular factors or a unique interaction between Nef and a macrophage signal transduction pathway. Since Nef is a nonnuclear protein, an indirect pathway of action seems likely. The failure of *nef* to *trans* activate the urokinase plasminogen activator promoter, which is phorbol myristate acetate responsive in RAW264 cells (3), at least argues against the possibility that *nef* action involves protein kinase C activation. Clear evidence that Nef does alter cell function is seen in its cytostatic activity (Table 4). Unfortunately, the cytostatic activity militates against the production of stably transfected cell lines expressing high levels of *nef* mRNA or protein, and further progress will require the use of a vector system with an inducible promoter.

Both the *trans*-activating effects of *nef* and its cytotoxic and cytostatic effects on RAW264 cells were unaffected by a frameshift mutation at amino acid 35, suggesting that either the mRNA or the N-terminal amino acids of *nef* have biological activity. Given the tight preservation of the *nef* reading frame in natural HIV variants (23), assignment of a distinct biological activity to the entire protein seems compelling. In SIV in vivo, there appears to be strong selective pressure in favor of functional reversion of stop mutations in *nef* (17). One possibility is that the intact protein serves as a decoy antigen, released from dying T lymphocytes but not expressed in a functional manner on the surface of infected macrophages. It would be of some interest to ascertain whether Nef-specific cytotoxic T cells can kill HIV-infected macrophages. Conversely, there is other evidence that the N terminus of Nef contains critical amino acids involved in its biological activity. Luria et al. (21), in a study showing that *nef* overexpression blocked the function of the interleukin-2 promoter in T cells, noted that substitutions at positions 15, 29, and 33, all upstream of the frameshift in the *nef*-fs gene used in our study, abolished this *nef* activity. The variant carrying these mutations was referred to as Nef-2. While having no inhibitory effect on the interleukin-2 promoter, Nef-2 was able to compete in *trans* with the inhibitory effect of the functional *nef* gene product Nef-1. These observations would support the proposal that the *nef* gene product has at least two functional domains, at least one of them residing in the N terminus.

nef mRNA is the most abundant of the small multiply spliced transcripts from HIV-1 infected cells, and differently spliced species appear with characteristic frequency in T cells and macrophages (26). A role for *nef* mRNA, rather than its

translation product, in some of the actions of the expression plasmids is apparently favored by the data in Table 4. Linearization of the parent and frameshift *nef* expression plasmids with *Kpn*I at bp 226 abolished the cytostatic activity of the plasmid. Conversely, linearization immediately upstream of the polyadenylation signal with *Bst*EII increased the cytostatic activity. Although these two digests are important controls assuring us that cytostasis is not due to poison sequences in the *nef* DNA, they do not necessarily imply that protein or RNA sequences downstream of the *Kpn*I site are essential to Nef function. We have no way of ascertaining whether the cells transfected with *Kpn*I-digested plasmid express a Nef protein truncated at amino acid 73. Analysis of the *nef* sequences using the SEQL program on the ANGIS (Australian National Genome Information Service) computer reveals a number of stable single-hairpin-loop RNA structures. Several have free energy values similar to that of the stable stem-loop structure formed by TAR RNA, especially structures between bases 8530 to 8624 and 8731 to 8831. The removal of these structures could alter the stability of the resultant *nef* mRNA, so that it is rapidly degraded or differently processed, resulting in altered production of Nef protein. If the *nef* mRNA is involved in the responses that we have documented, the long stable stem-loop structures referred to above could play a role, being perceived by the cell as double-stranded RNA. Macrophages respond to intracellular polyribonucleotides by producing alpha interferon (15). This process is primarily an antiviral defense which entails numerous physiological consequences, including cessation of cell proliferation. Alpha interferon is indeed induced in HIV-infected unstimulated human monocyte/macrophage cells following replication of the virus (30).

In summary, we have suggested a positive role for the *nef* gene product in regulation of HIV transcription in macrophages and a negative effect of the same product on cell growth and proliferation. The combined effect of these activities may help to subvert the macrophage from growth and the production of its own gene products to the production of viral gene products, a strategy common to many viruses.

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